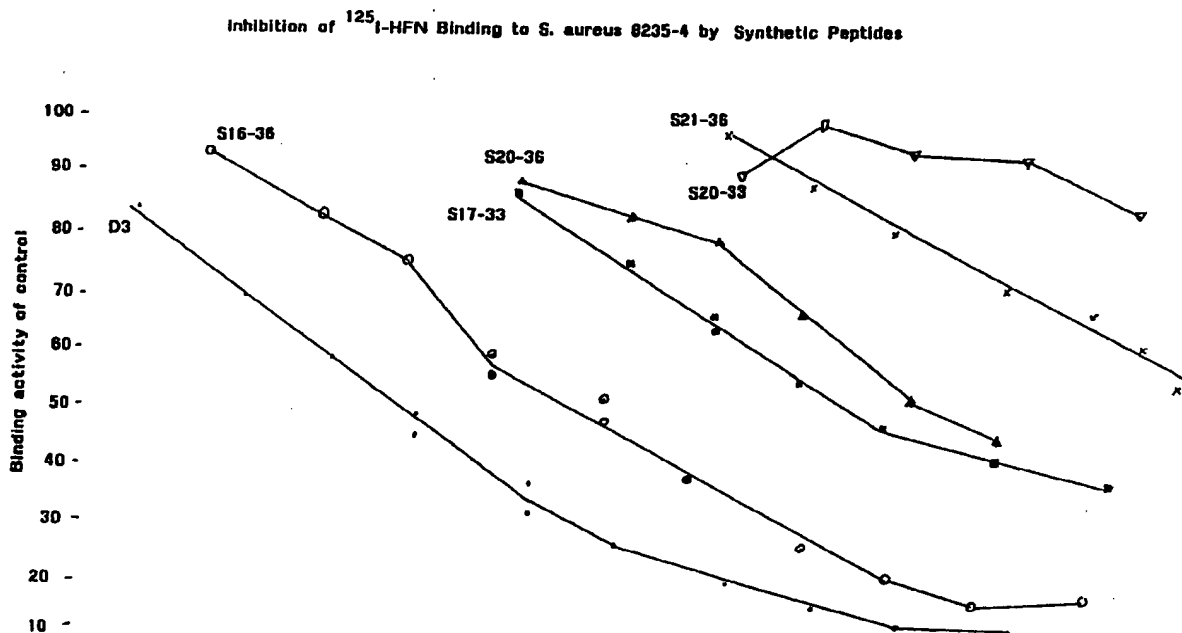




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(71) Applicant (for all designated States except US): ALFA-LAVAL AGRI INTERNATIONAL AKTIEBOLAG [SE/SE]; Box 39, S-147 00 Tumba (SE).		Published With international search report.	
(72) Inventors; and (75) Inventors/Applicants (for US only) : HÖÖK, Magnus [SE/US]; 129 Stevens Hill Circle, Birmingham, AL 35244 (US). McGAVIN, Martin [US/US]; 1717 Beacon Crest Circle, Birmingham, AL 35209 (US). RAUCCI, Giuseppe [IT/IT]; Menarini Recherche Sud Spa, Biotechnolgy Dept., Via Tito Speri, 10, I-00040 Pomezia (IT).			

(54) Title: A FIBRONECTIN BINDING PEPTIDE



(57) Abstract

The present invention relates to a fibronectin binding peptide having the structure R'-PSYQFGGHNS VDFEEDT-R<sup>2</sup>, wherein R' is hydrogen of K or DK, and R<sup>2</sup> is hydroxy, L, LP, or LPK.

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A FIBRONECTIN BINDING PEPTIDEDESCRIPTIONTechnical field

5 The present invention relates to a fibronectin binding peptide.

The object of the present invention is to obtain a minimal fibronectin binding polypeptide.

10

A further object is to obtain a possibility of preparing said peptide by chemical synthesis.

Further objects will be apparent from the following  
15 description.

Background of the invention

WO-A1-85/05553 discloses bacterial cell surface proteins having fibronectin, fibrinogen, collagen, and/or laminin  
20 binding ability. Thereby it is shown that different bacteria have an ability to bind to fibronectin, fibrinogen, collagen, and/or laminin. It is further shown that fibronectin binding protein has a molecular weight of 165 kD and/or 87 kD, whereby it is probable that the smaller protein  
25 is a part of the larger one.

Fibronectin is a disulfide linked dimeric glycoprotein ( $M_r$  450,000) which is present in a soluble form in blood plasma and other body fluids, and is deposited in a fibrillar form as a major constituent of the extracellular matrix  
30 of loose connective tissue. It is composed of three different structural motifs, termed type I, II, and III homologues (Petersen et al., 1983; PNAS), resulting in a modular organization of the fibronectin molecule in which its several  
35 biological activities can each be attributed to specific domains.

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The major biological role of fibronectin appears to be related to its ability to mediate the adhesion of eukaryotic cells to the extracellular matrix through a specific interaction between discrete cell surface receptors, and a  
5 105-kDa central region of the molecule composed entirely of type III homology units (Pierschbacher and Ruoslahti, 1984, Nature 309:30-33). The cell binding domain is succeeded at its C-terminal end by a 31 kDa heparin binding domain and a 30 kDa fibrin binding domain. Preceding the cell binding  
10 domain is a 42 kDa gelatin (collagen) binding domain, and an N-terminal 29 kDa domain consisting of five consecutive type I repeats that binds fibrin, heparin and bacteria (Yamada 1983, Ann. Rev. Biochem. 52:761-799).

15 Several pathogenic Gram-positive staphylococci and streptococci have been reported to bind exclusively to the 29-kDa N-terminal domain of fibronectin (Speziale et al., 1984, J. Bacteriol. 157:420-427, Mosher and Proctor, 1980 Science 209:927-929). Some of the Gram-negative enteric bacteria,  
20 namely Escherichia coli, Salmonella typhimurium, S. enteritidis, and S. dublin have also been reported to bind fibronectin (Froman et al., 1984, JBC 259:14899-14905; Van de Water et al., 1983, Science 220:201-204; Faris et al., 1986, FEMS Microbiol. Lett. 34:221-224; Baloda et al.,  
25 1986, FEMS Microbiol. Lett. 34:225-229; Baloda et al., 1985, FEMS Microbiol. Lett. 28:1-5; Kristiansen et al., 1987), and it was reported for E. coli, that the bacteria bound to the 29 kDa N-terminal domain, and a second unidentified site (Froman et al., 1984). In contrast, the  
30 spirochete Treponema palladium, and the trypanosome Trypanosoma cruzi differ from other fibronectin binding microorganisms in showing a specificity for the 105 kDa eukaryotic cell binding domain (Thomas et al., 1985a, J. Exp. Med. 161:514-525; Ouaisi et al., 1986, J. Exp.  
35 Med. 162:1715-1719).

The dissociation constants for the interaction of these microorganisms with fibronectin show a  $10^2$  to  $10^3$ -fold higher affinity than that reported for the binding of intact fibronectin to human fibroblasts (Hook *et al.*, 1989; Akiyama and Yamada, 1985, JBC 260:4492-4500). This avidity for fibronectin may be a virulence factor, facilitating the colonization of wound tissues and blood clots, where a fibronectin rich matrix is known to be deposited during the first and second weeks of wound repair (Kurikinen *et al.*, 1980, Lab Invest. 43:47-51; Grinell *et al.*, 1981, J. Invest. Dermatol. 76:181-189; Clark *et al.*, 1982, J. Invest. Dermatol. 79:269-276). It has been the intent of the present research to characterize microbial fibronectin binding proteins (FnBPs), and to develop receptor analogs for potential therapeutic application towards preventing or reducing the risk of wound infection.

Studies on FnBPs have so far mostly dealt with the FnBP of S. aureus. A FnBP with an  $M_r$  of 210,000 has been reported from three laboratories (Espersen and Clemmensen, 1982, Infect. Immun. 37:526-531; Froman *et al.*, 1987, J. Biol. Chem. 262:6564-6571; Signas *et al.*, 1989, PNAS 86:699-703). The gene encoding the FnBP of S. aureus 8325-4 has been cloned and expressed in E. coli (Flock *et al.*, 1987, EMBO J. 2351-2357). The fibronectin binding activity was localized to a 600 base pair insert, which when fused in frame with DNA coding for two IgG binding domains of staphylococcal protein A, yielded a protein fusion designated ZZ-FR which had the same fibronectin binding activity as the native 210 kDa receptor. Subsequent DNA sequence analysis revealed the 600 base pair insert to code for 184 amino acids, of which a prominent feature was a 38 amino acid homology unit repeated three times, and partially a fourth (Signas *et al.*, 1989).

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It has been found that synthetic peptide analogs to each of the homology units, designated D1, D2, and D3, were effective inhibitors of fibronectin binding to S. aureus 8325-4. Peptide D3 constructed on the third homology unit was 50 to 100-fold more effective as an inhibitor of fibronectin binding, but showed a considerable deviation from the basic homology unit. In the present invention it is further defined the fibronectin binding determinant within the D3 homology unit through means of chemical modification, proteolytic cleavage, and chemical synthesis of a number of smaller peptides encompassing the D3 sequence.

Description of the present invention

The present invention relates to a minimum fibronectin binding peptide having the structure

$R'-PSYQFGGHNSVDFEEDT-R^2$

wherein  $R'$  is hydrogen, K or DK, and  $R^2$  is hydroxy, L, LP, or LPK

whereby

20	A	Ala, Alanine
	R	Arg, Arginine
	N	Asn, Asparagine
	D	Asp, Aspartic acid
	C	Cys, Cysteine
25	C	Cys, Cystine
	G	Gly, Glycine
	E	Glu, Glutamic acid
	Q	Gln, Glutamine
	H	His, Histidine
30	I	Ile, Isoleucine
	L	Leu, Leucine
	K	Lys, Lysine
	M	Met, Methionine
	F	Phe, Phenylalanine
35	P	Pro, Proline
	S	Ser, Serine

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W Trp, Tryptophan

Y Tyr, Tyrosine

V Val, Valine

5

MATERIALS AND METHODSBacteria and Growth Media

Staphylococcus aureus 8325-4, described by Lofdahl et al., (1983, PNAS USA 80: 697-701) was stored in deep agar at 4°C. Cultures were started by inoculation into Brain Heart Infusion broth (Difco, Detroit, MI). After overnight incubation at 37°C, the bacteria were collected by centrifugation, suspended in phosphate buffered saline (pH 7.4), containing 0.02% (wt/vol) sodium azide, and adjusted to a value of  $10^{10}$  cells/ml of suspension by reference to a standard curve relating cell count to optical density. The cells were then heat killed at 88°C for 20 minutes, aliquoted, and stored frozen at -20°C.

20 Preparation and Iodination of Ligands

Human fibronectin was either purchased from the New York Blood Center, or purified from outdated plasma obtained from the same source, as described by Engvall and Rouslahti (1977, Int. J. Cancer Res. 20:1-5). For purification of the 29-kDa N-terminal domain of fibronectin, human fibronectin was diluted to 1 mg/ml in a buffer consisting of 25 mM Tris-HCl (pH 7.6), 50 mM sodium chloride, 2.5 mM calcium chloride, and 0.5 mM ethylenediamine tetraacetic acid (EDTA). Protease digestion was initiated by addition of 5 µg of thermolysin (Calbiochem, La Jolla, CA) for each 1 mg of fibronectin, followed by incubation for 2 hours at room temperature with end over end mixing. The reaction was stopped by addition of EDTA to 5 mM. The 29 kDa fragment was then isolated by passage of the digest through an affinity gel matrix consisting of ZZ-FR fusion protein (Flock et al., 1987) coupled to Sepharose CL-4B (Pharmacia,

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Uppsala, Sweden), and subsequently eluted with 4M guanidine hydrochloride in phosphate buffered saline (PBS). The eluate was then dialyzed extensively against PBS. Iodination of either fibronectin or the 29 kDa fragment was conducted according to the chloramine T protocol of Hunter (1978).

#### Assay of Fibronectin Binding

Synthetic peptides were analyzed for fibronectin binding activity by measuring their ability to compete with cells of S. aureus 8325-4 for binding of  $^{125}\text{I}$ -labeled fibronectin, or labeled N-terminal 29 kDa fragment, exactly as described previously (Signas et al., 1989). Where indicated, this assay was modified by replacing the bacterial suspension with 40 l of the ZZ-FR Sepharose (coupling ration of 0.3 mg/ml of swollen gel). In this case, the background radioactivity was determined from an assay in which unmodified Sepharose was used in place of the affinity matrix. Radiolabeled ligand bound either to bacteria or ZZ-FR Sepharose was quantitated in an LKB gamma counter (Turku, Finland).

#### Synthesis and Purification of Peptides

All peptides with the exception of S16-36 were synthesized on an Applied Biosystems automatic peptide synthesizer at the University of Alabama at Birmingham Cancer Center core facility as described previously (Signas et al., 1989). Peptide S16-36 was synthesized by Vega Biotechnologies (Tucson, AZ) using an F-moc synthesis procedure. Peptides were purified from the crude preparations by reverse-phase HPLC using a preparative scale  $\text{C}_{18}$  column (Vydac 218TP510; The Separation Group, Hesperia, CA) and an LKB HPLC system. The buffer used for the purification was TEAP/EDTA (pH 5.5), consisting of 0.11% (vol/vol) phosphoric acid, 0.28% (vol/vol) triethylamine, and 0.25 mM EDTA. The elution buffer was 15% of TEAP/EDTA in acetonitrile.



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Triethylamine was purchased from Pierce (Rockford, IL), while HPLC grade phosphoric acid and acetonitrile was from Fisher (Pittsburgh, PA). After reverse-phase chromatography, the appropriate fractions were dialyzed extensively against 50 mM ammonium bicarbonate and lyophilized. The purity of the resulting preparations was checked by reverse-phase chromatography on an analytical C<sub>18</sub> column (Vydac 218TP546; The Separation Group, Hesperia, CA) using a buffer consisting of 0.1% (vol/vol) trifluoroacetic acid (Pierce; Rockford, IL) in water, and 0.1% (vol/vol) TFA in 60% acetonitrile as the elution buffer. Peptides eluting in this buffering system were dried by roto-evaporation and submitted for either N-terminal sequence analysis or amino acid composition analysis.

Peptide sequencing was done by the Protein Chemistry Core of the University of Alabama at Birmingham on an Applied Biosystems peptide sequencer, Model 470A. The amino acid analysis was done by the Atherosclerosis Research Unit Protein Chemistry Core.

#### Proteolytic Digestion of Synthetic Peptides

L-(tosylamido-2-phenyl)-ethyl-chloromethyl-ketone (TPCK) treated trypsin (code TRTPCK) and chymotrypsin (code CDS) was obtained from Worthington (Freehold, NJ). Soybean trypsin inhibitor and TPCK were purchased from Sigma (St. Louis, MO), endoproteinase Glu-c (V8 protease) was purchased from Boehringer Mannheim and lysine endopeptidase C (LEC) was obtained from Calbiochem (La Jolla, CA).

For both trypsin and chymotrypsin digestion, peptide D3 was dissolved at 2 mg/ml in 0.1M ammonium bicarbonate and treated with protease at an enzyme to substrate ratio of 1:200 for 24 h at 37°C. Trypsin was inactivated by addition of soybean trypsin inhibitor and phenylmethylsulfonylfluoride to 20 g/ml and 1 mM, respectively. For

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chymotrypsin, TPCK and PMSF were added to achieve concentrations of 0.3 mg/ml and 1 mM, respectively.

- For endoproteinase Glu-c digestion, the enzyme to substrate ratio was 1:100 and peptide was 2 mg/ml in phosphate buffered saline. After incubation for 24h at room temperature, the digestion was stopped by addition of PMSF to 1 mM. For LEC digestion, peptide was dissolved in PBS at 2 mg/ml, to which was added 1 unit of protease per ml.
- 10 Digestion was allowed to continue for 40 h at 37°C, and was terminated when the cleavage products were separated by HPLC. For each digestion, reverse-phase HPLC with the TEAP/EDTA buffer system was used to monitor the progress of proteolysis and to purify the cleavage products. Samples
- 15 needed for composition analysis or N-terminal sequencing were further chromatographed with the TFA buffer system.

#### Chemical Modification of Synthetic Peptides

- Dihydroxypropylation-reduction of the amino side chain of lysine was a modification of the method of Acharya (1984; J. Chrom. 297:37-38). Peptide was dissolved in PBS (2.5 mg/ml), followed by addition of DL-glyceraldehyde and sodium cyanoborohydride (Sigma, St. Louis, MO) to a final concentration of 0.1 and 1.0 molar, respectively. The
- 25 reaction was conducted at room temperature for 1 h and was stopped by dialysis against 50 mM ammonium bicarbonate. The extent of derivatization of lysine side residues was determined by amino acid analysis.
- 30 The carboxylic side chains of glutamic and aspartic residues were converted to glycine methyl esters by 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide (EDC) mediated condensation (Lungblad and Noyes, 1985; Chemical Reagents for Protein Modification, Vol II). Peptide was dissolved in
- 35 double distilled water at 2 mg/ml and rendered 1M in glycine methyl ester (Sigma). The pH was adjusted to 4.3

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with 0.1M HCl and EDC was added to a concentration of 0.1M. The solution was mixed for 3 h at room temperature with adjustment of pH back to 4.3 after 30 min. and again after 1.5 h. The reaction was stopped by dialysis against PBS.

5 The degree of derivatization was determined by the increase of the glycine molar ratio as compared to the underivatized peptide. A derivatization was done in which ethanolamine was used in place of glycine methyl ester, and a control experiment was performed in which only EDC was mixed with  
10 the peptide.

The phenyl side chains of tyrosine residues were oxidized by addition of 10  $\mu$ l of tetranitromethane (TNM; Sigma) to each ml of 1 mg/ml peptide dissolved in 100 mM Tris-HCl,  
15 150 mM NaCl (pH 8.0), and mixing for 1 hr at room temperature, followed by dialysis against Tris/NaCl buffer (pH 8.0) to remove TNM.

The amino acid sequence of the three D-repeats is shown in  
20 Figure 1. The D1 and D2 repeats show a high degree of homology differing in only 5 of 38 residues (87% homology), whereas D3 shows less than 50% homology with D2. However, as noted previously (Signas *et al.*, 1989), the peptide affected a 50% inhibition of  $^{125}$ I-HFN binding to  
25 cells of *S. aureus* 8325-4 at a concentration of 2 g/ml, while concentrations of 90 g and 230 g/ml for D2 and D1, respectively, were required to give the same degree of inhibition. We, therefore, chose peptide D3 for further  
characterization.

30

#### Chemical Modification

Table 1 is composed on the data from amino acid composition analysis of peptide D3 and its derivatization products. Dihydroxypropylation of lysine side residues was quantitative-  
35 ly complete showing a greater than 98% reduction in lysine content in comparison to D3. A minor side reaction normally

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occurring through use of this protocol was the derivatization of phenylalanine in position 1 via its free NH<sub>2</sub> group, as noted by a slight decrease in phenylalanine content. Conversion of aspartic and glutamic residues to glycine methyl-ester was also quantitatively complete, as noted by the change in glycine content from 54 residues per 1,000 to 293. This is the value expected for the 2 glycine residues in D3, and the 9 additional residues which were added by complete chemical modification of the aspartic/glutamic residues. The TNM mediated oxidation of tyrosine was also successful showing a 95% reduction in tyrosine content.

Figure 2 shows the inhibition activity of the chemically modified peptides compared to that of unmodified D3. While lysine and tyrosine modifications only partially diminished the activity of D3, modification of aspartic/glutamic residues resulted in a total loss of activity. This result was observed with either glycine methyl ester or ethanolamine condensation on the carboxylic side chains. As a control, EDC treatment alone showed only a small loss in activity similar to that observed with the lysine and tyrosine modifications. The observed loss of activity is therefore due specifically to modification of carboxyl side chains.

Reverse-phase HPLC of trypsin, chymotrypsin and endoprotease Glu-c digests of D3 confirmed that the peptide substrate was completely converted into smaller products. Representative reverse-phase profiles of D3 and its' tryptic digest are shown in Figure 3, along with the identity of the fragments which were subsequently purified. Initial assays were conducted with the crude digests in the presence of appropriate protease inhibitors to test for binding of either <sup>125</sup>I-HFN or the <sup>125</sup>I-29 kDa N-terminal fragment to an S. aureus cell suspension (Fig. 4). After complete trypsin digestion, as verified in Fig. 3, the resulting unfractionated cleavage products showed little loss in bio-

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logical activity, whereas the chymotrypsin digest showed no inhibitory activity. The results were less clear with the endoprotease Glu-c digest which showed inhibitory activity using  $^{125}\text{I}$ -29 kDa as the ligand, but none with  $^{125}\text{I}$ -HFN. This may be due to changes in conformation when the N-terminal domain is detached by proteolytic cleavage.

Figure 1 shows the amino acid sequences of several cleavage products which were purified from the crude digests by reverse-phase HPLC. The major cleavage product purified from the trypsin digest constituted residues 15-36 (T15-36) of the 37 amino acid D3, and biological activity was retained by this peptide (Fig. 5). Further digestion of this peptide with lysine endopeptidase-c resulted in the removal of an N-terminal dipeptide producing TL17-36 which still showed inhibitory activity. Cleavage of T15-36 with endoprotease Glu-c resulted in removal of a C-terminal hexapeptide and concomitant loss of activity. The sequences of peptides isolated from endoprotease Glu-c and chymotrypsin digests of D3 are also given in Fig. 1. None of these peptides had inhibitory activity (Fig. 5).

As T15-36 showed biological activity similar to that of D3, several synthetic peptides spanning this sequence were constructed (Fig. 2) and tested for biological activity (Fig. 6). Peptide S16-36, one residue shorter at the N-terminus than T15-36, elicited a 50% inhibition of fibronectin binding at a concentration of 10 nmol/ml compared to 3 nmol/ml for D3. Knowing that peptide TL17-36 retained biological activity, we constructed peptide S17-33 to test for the involvement of the C-terminal amino acids in fibronectin binding. It was found to have a markedly reduced but consistently observed biological activity, eliciting a 50% inhibition of fibronectin binding at a concentration of 100 nmol/ml. Further reduction in size of this peptide from

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the N-terminus producing S20-33 resulted in a near total loss of activity. However, expansion of peptide S20-33 from the C-terminus to produce S20-36 resulted in a peptide which had a level of activity comparable to that of S17-33, showing 50% inhibition at 200 nmol/ml. Peptide S21-36 still showed inhibition activity which was directly proportional to the amount of peptide, but was about 10-fold less effective than S20-36. We have, therefore, reduced the fibronectin binding region of D3 to a core peptide, S20-33, which is essential for activity but alone is not sufficient to inhibit binding. Addition of the sequence PSY to the N-terminus or LPK to the C-terminus resulted in peptides with similar biological activity. Any further reduction in the size of these peptides causes a large reduction in activity.

15

Adhesion of bacteria to fibronectin has been proposed as a virulence factor enabling bacteria to colonize wound tissues and blood clots (Ryden et al., 1983, JBC 258: 3396-3401; Proctor et al., 1982, JBC 257: 14788-14794); and the prevention of this interaction is an attractive target for either prophylaxis or vaccination studies. Some progress towards achieving this goal has been reported previously from studies of fibronectin binding by Treponema palladium (Thomas et al., 1985, J. Exp. Med., 14-25) and Trypanosoma cruzi. A monoclonal antibody that inhibits attachment of eukaryotic cells to fibronectin coated substrates also blocks the binding of fibronectin to Treponema palladium (Thomas et al., 1985a) and Trypanosoma cruzi (Ouaissi et al., 1986; J. Exp. Med., 162: 1715-1719). Binding was also prevented by the peptide RGDS, the amino acid sequence within the cell binding domain of fibronectin which is recognized by the eukaryotic fibronectin integrin receptors (Thomas et al., 1985b; J. Exp. Med. 162: 1715-1719; Ouaissi et al., 1986). This same peptide also blocked the attachment of spirochetes to cultured human cells. However, it must be considered that the RGDS peptide would also abrogate the

35

role of fibronectin. Secondly, these microorganisms are unusual in their specificity for the cell binding domain of fibronectin.

- 5 The approach has been to characterized the interaction between fibronectin and the S. aureus receptor at the molecular level through the use of synthetic peptides.

- Results from chemical modification of the D3 peptide  
10 indicate that glutamic and aspartic residues are essential for binding to fibronectin. This is of particular interest, as the motif of either FEEDT or DFEEDT appears in each of the three D-repeats of the S. aureus FN-receptor. In fact, in D3 this motif is repeated twice. Furthermore, sequence  
15 data obtained from genes coding for two fibronectin receptors of Streptococcus dysgalactiae each show three repeat sequences containing either DFTEEDT (gene I) or EVEDT (gene II) motifs in each of the repeats (submitted for publication). Loss of activity upon cleavage with endoprotease  
20 Glu-c may be attributed to disruption of the FEEDT motif, and is further indication of the involvement of acidic amino acid residues.

- The activity showed by the D3 tryptic digest product T15-36,  
25 and synthetic peptide S16-36 effectively localized the fibronectin binding determinant of D3 to the C-terminal half of the peptide which contains the FEEDT motif. It is interesting that the sequence IEEDT appears also near the N-terminus of D3 (residues 8-12) and that repeats D1 and D2  
30 contain FEEDT at the same location. The presence of this motif repeated twice in D3 may account for its substantially higher biological activity in comparison to D1 and D2.

- Attempts to further localize the fibronectin binding determinant within peptide S16-36 resulted in substantial losses  
35 of biological activity. We believe that peptide S20-33

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contains the amino acid residues which establish contact with and bind to fibronectin. This peptide by itself shows essentially no activity. However, addition of the tripeptide PSY to the N-terminus (S17-33) or LPK to the C-terminus (20-36) resulted in a dramatic recovery of activity. If this core peptide were to have both flanking sequences, it would be one residue shorter at the N-terminus than peptide S16-36 which had a level of activity directly comparable to D3. Our chemical modification experiments indicated that neither tyrosine, nor lysine are involved in contacts with fibronectin. We propose that the PSY and LPK flanking tripeptides are not involved in contacts with fibronectin, but are required for the peptide to assume a proper and stable conformation. We note that loss of one amino acid from the N-terminus of S20-36 caused a greater than 10-fold reduction of activity.

The previous study of D3 and the ZZ-FR fusion protein (Signas et al., 1989), in combination with the present results, indicate that while a dramatic drop of activity occurs when the continuity of the three tandem D-repeats is disrupted, the change in activity when D3 is reduced to S17-36 is quite irrelevant. Further reduction in the size of S17-36 causes a loss in activity comparable to that observed through physical separation of the tandem repeats. Therefore, the secondary structure of the individual repeats provides one level of affinity for fibronectin, and the tertiary organization of the three repeats results in an affinity which is an order of magnitude higher. A third, hypothetical and as of yet unexplored, level of organization and further increase in affinity for fibronectin may be attained through the quaternary organization of fibronectin receptors on the cell surface of S. aureus 8325-4.



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Table 1. Amino Acid Composition of Peptide D3, and of its'  
Derivatization Products

Amino acid residues per 1,000

	Unmodified	DHP-K <sup>1</sup>	Gly-OCH <sup>2</sup> <sub>3</sub>	TNM-Y <sup>3</sup>
5				
Asx*	217.7 (189.2)	226.8	155.9	157.1
Glx*	156.3 (162.2)	193.4	138.4	174.9
Ser	51.4 (54.1)	58.7	44.7	57.7
10 Gly*	54.6 (54.1)	62.7	292.7	64.6
His	57.4 (54.1)	65.8	43.5	46.6
Arg	-	-	-	-
Thr	82.7 (81.1)	95.3	76.9	100.6
Ala	-	-	-	-
15 Pro	54.5 (54.1)	63.5	49.9	60.4
Tyr*	18.2 (27.0)	23.4	10.5	0.9
Val	51.6 (54.1)	61.6	39.3	57.1
Met	-	-	-	-
Cys	-	-	-	-
20 Ile	29.3 (54.1)	33.8	26.8	31.8
Leu	30.6 (27.0)	33.5	22.2	32.8
Phe	70.2 (81.1)	55.0	57.6	72.1
Lys*	107.9 (108.1)	2.4	89.2	100.9

25 Amino acids either targeted for, or affected by, chemical modification are indicated by asterisks. Numbers in brackets refer to the expected values, based on the known comparison of D3.

30 <sup>1</sup> Dihydroxypropylation - reduction of lysine

<sup>2</sup> Conversion of aspartic and glutamic residues to glycine methyl esters

35 <sup>3</sup> Tetranitromethane oxidation of tyrosine

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The present fibronectin binding peptide can be used for immunization, whereby the peptide, preferably in combination with a fusion protein to create a large antigen to respond to, is injected in dosages causing immunological reaction in the host mammal. Thus the fibronectin binding peptide can be used in vaccination of ruminants against mastitis caused by Staphylococcal infections.

Further, the fibronectin binding peptide can be used to block an infection in an open skin wound by wound treatment using the fibronectin binding peptide in a suspension. Thus the fibronectin binding peptide can be used for the treatment of wounds, e.g. for blocking protein receptors, or for immunization (vaccination). In the latter case the host body produces specific antibodies, which can protect against invasion of bacterial strains comprising such a fibronectin binding peptide. Hereby the antibodies block the adherence of the bacterial strains to damaged tissue.

Examples of colonizing of a tissue damage are:

- a) colonizing of wounds in skin and connective tissue, which wounds have been caused by a mechanical trauma, chemical damage, and/or thermal damage;
- b) colonizing of wounds on mucous membranes, such as in the mouth cavity, or in the mammary glands, urethra, or vagina;
- c) colonizing on connective tissue proteins, which have been exposed by a minimal tissue damage (microlesion) in connection with epithelium and endothelium (mastitis, heart valve infection, hip exchange surgery).

30

When using the present FNBP, or the peptide, for the purpose of immunization (vaccination) in mammals, including man, the peptide is dispersed in sterile, isotonic saline solution, optionally while adding a pharmaceutically acceptable dispersing agent. Different types of adjuvants can further be used in order to sustain the release in the tissue, and thus

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expose the peptide for a longer time to the immunodefense system of a body.

- 5 A suitable dosage to obtain immunization is 0,5 to 5 ug of peptide, per kg bodyweight and injection of immunization. In order to obtain a durable immunization, vaccination should be carried out at more than one consecutive occasions with an interval of 1 to 3 weeks, preferably at three occasions.
- 10 When using the present peptide, for topical, local administration the protein is dispersed in an isotonic saline solution to a concentration of 25 to 250 ug per ml. The wounds are then treated with such an amount only to obtain a complete wetting of the wound surface. For an average wound
- 15 thus only a couple of millilitres of solution are used in this way. After treatment using the peptide solution the wounds are suitably washed with isotonic saline or another suitable wound treatment solution.
- 20 Further the fibronectin binding peptide as well as the minimal fibronectin binding site peptide, of the present invention can be used to diagnose bacterial infections caused by Staphylococci strains, whereby a fibronectin binding peptide of the present invention is immobilized on a
- 25 solid carrier, such as small latex or Sepharose<sup>®</sup> beads, whereupon sera containing antibodies are allowed to pass and react with the FNBP thus immobilized. The agglutination is then measured by known methods.
- 30 Further, the peptide can be used in an ELISA test (Enzyme Linked Immuno Sorbent Assay; E Engvall, Med. Biol. 55, 193, (1977)). Hereby wells in a polystyrene microtitre plate are coated with the FNBP, and incubated over night at 4°C. The plates are then thoroughly washed using PBS containing 0.05%
- 35 TWEEN 20, and dried. Serial dilution of the patient serum were made in PBS-Tween, were added to the wells, and in-

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5 cubated at 30°C for 1.5 hrs. After rinsing antihuman-IgG conjugated with an enzyme, or an antibovine-IgG conjugated with an enzyme, respectively, horse radish peroxidase or an alkaline phosphatase, was added to the wells and incubated  
10 at 30°C for 1,5 hrs, whereupon when the IgG has been bound thereto, and after rinsing, an enzyme substrate is added, a p-nitrophosphate in case of an alkaline phosphatase, or ortophenylene diamine substrate (OPD) in case a peroxidase has been used, respectively. The plates comprising the wells were thus then rinsed using a citrate buffer containing 0.055% OPD, and 0.005% H<sub>2</sub>O<sub>2</sub>, and incubated at 30°C for 10 min. Enzyme reaction was stopped by adding a 4N solution of H<sub>2</sub>SO<sub>4</sub> to each well. The colour development was measured using a spectrophotometer.

15

Depending on the type of enzyme substrate used a fluoroscense measurement can be used as well.

20 Another method to diagnose Staphylococci infections is by using the DNA gene probe method based on the peptide sequence. Thereby the natural or synthetic DNA sequences are attached to a solid carrier, such as a polystyrene plate as mentioned above, by e.g. adding a milk in the case of diagnosing a mastitis, to the surface. The DNA gene probe,  
25 optionally labelled enzymatically, or by a radioactive isotope is then added to the solid surface plate comprising the DNA sequence, whereby the DNA gene probe attaches to the sequence where appearing. The enzyme or the radioactive isotope can then readily be determined by known methods.

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FIGURE LEGENDSFigure 1

Amino acid sequence of the tandem repeats constituting the fibronectin binding site of the S. aureus 8325-5 FnBP. Synthetic peptides corresponding to each of the repeats have been named D1, D2, and D3. Beneath the sequence of D3 is illustrated the composition of several proteolytic cleavage products of the D3 peptide, and of smaller synthetic peptides which were used to further define the fibronectin binding determinant of D3. A two number preceded by one or two letters is used to identify the individual peptides. The numbers refer to the position of the N- and C-terminal residues of each peptide within the 37 amino acid sequence of D3. The letters V, C, and T indicate cleavage products isolated from endoprotease Glu-c, trypsin, or chymotrypsin digests, respectively, of peptide D3. Peptide T15-36 was subjected to a second digestion with either lysine endopeptidase-c producing TL17-36, or with endoprotease Glu-c producing TV15-30. The letter S is used to designate peptides generated through chemical synthesis.

Figure 2

Effect of chemical modification on the ability of peptide D3 to inhibit binding of  $^{125}\text{I}$ -HFN to S. aureus 8325-4. The indicated amount of peptide was mixed with  $5 \times 10^8$  cells and  $5 \times 10^4$  cpm of  $^{125}\text{I}$ -HFN in an assay volume of 0.5 ml of PBX supplemented with 0.1% (wt/vol) bovine serum albumin, 0.1% (vol/vol) Tween 80, and 0.02% (wt/vol) sodium azide. After 60 min. of end-over-end mixing at room temperature, unbound fibronectin was diluted by addition of 3 ml of ice cold PBS containing 0.1% (vol/vol) Tween 80. After centrifugation at  $1,350 \times g$  for 20 min., the supernatant was aspirated and the radioactivity associated with the bacterial pellet was quantitated on a LKB gamma counter.

- 20 -

Panel A: Inhibitory activity of unmodified D3 (-), and after modification on tyrosine ( $\Delta$ - $\Delta$ ), or lysine (o-o) residues.

- 5 Panel B: Inhibitory activity of unmodified D3 (-), and after modification of carboxylic side residues with glycine methyl ester (o-o), ethanolamine ( $\Delta$ - $\Delta$ ), or the EDC reagent alone ( $\square$ - $\square$ ).

10

Figure 3

Reverse-phase HPLC of peptide D3 before (Panel A), and after (Panel B) digestion with trypsin. The chromatography was performed using a Vydac C<sub>18</sub> analytical column and TFA-

- 15 buffered acetonitrile as the eluent at a flow rate of 1 ml/min. The numbers above the peaks identify the first and last residues of the cleavage products within the sequence of D3. Panel C is a profile of trypsin alone.

20

Figure 4

Assay for ability of peptide D3 to inhibit binding of <sup>125</sup>I-HFN or <sup>125</sup>I-29 kDa fragment of HFN to

S. aureus 8325-4 either before ( $\Delta$ - $\Delta$ ), or after (o-o)

- 25 complete digestion with trypsin (A,D), chymotrypsin (B,E), or endoprotease Glu-c (C,F).

Figure 5

- 30 Assay for the ability of purified protease cleavage products to inhibit binding of <sup>125</sup>I-HFN to S. aureus 8325-4.

The <sup>125</sup>I-HFN bound by cells in the absence of peptide was designated as 100%, and binding in the presence of peptides was expressed relative to this value.

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Figure 6

Assay for ability of synthetic peptides to inhibit binding of  $^{125}\text{I}$ -HFN to cells of S. aureus 8325-4. Assay conditions were as described in the legend to Fig. 2. The sequences of the peptides are illustrated in Fig. 1. The results are expressed as a percentage of the fibronectin bound by cells in the absence of peptide.

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CLAIM

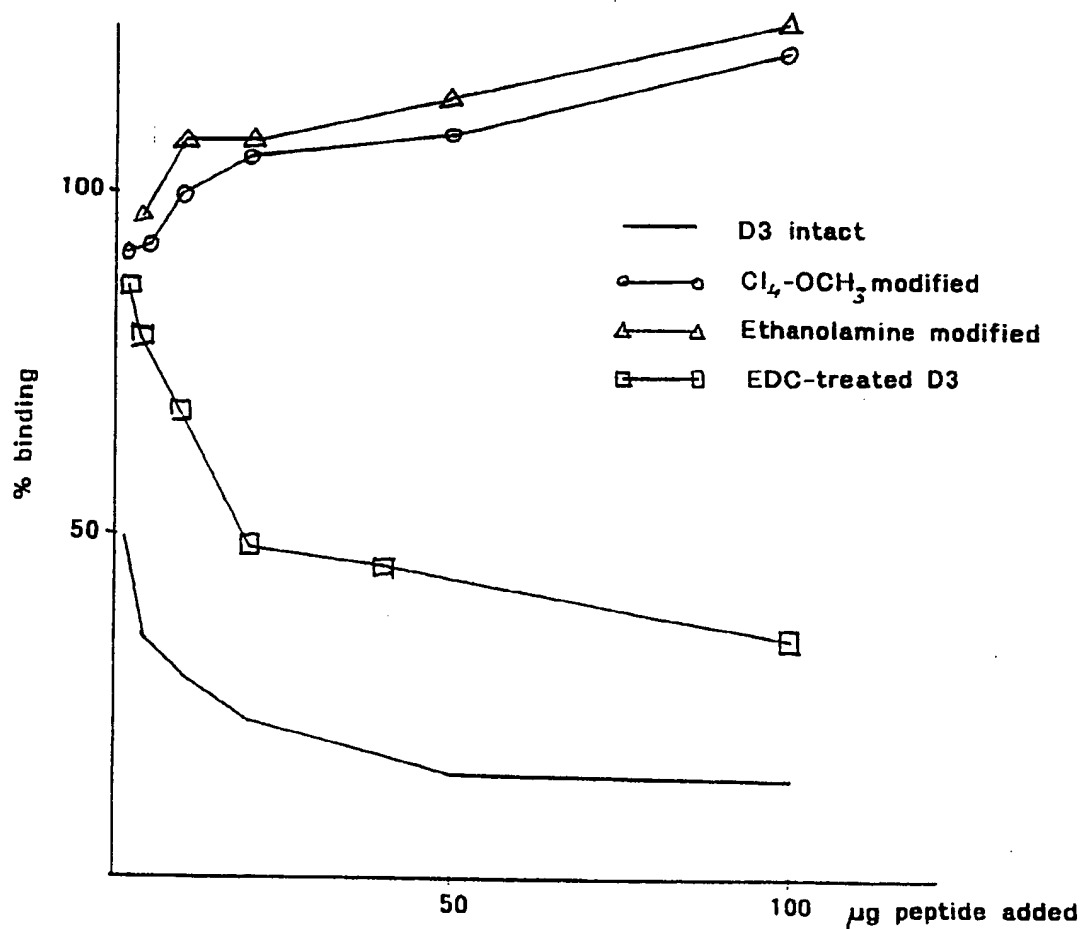
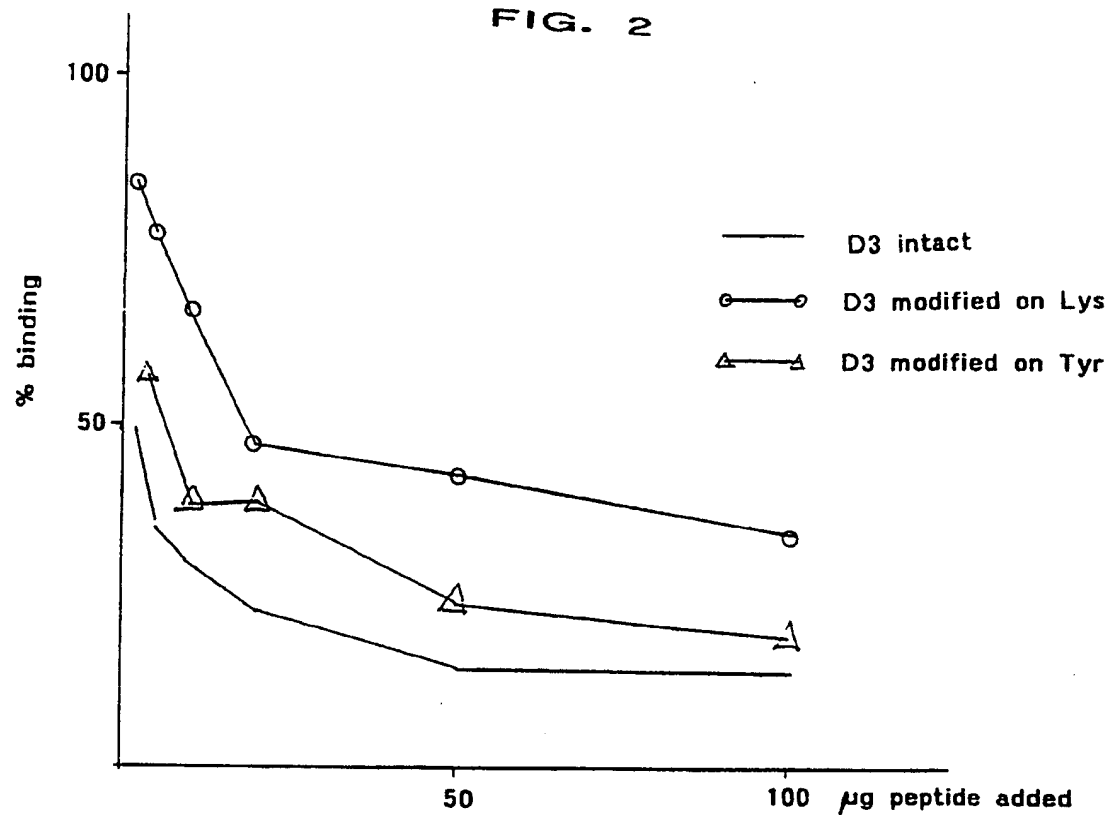
1.           Fibronectin binding peptide having the structure  
R'-PSYQFGGHNS VDFEEDT-R<sup>2</sup> wherein R' is hydrogen or K or  
5   DK, and R<sup>2</sup> is hydroxy, L, LP, or LPK.



D1	Q	N	S	G	N	Q	S	F	E	E	D	T	E	E	D	K	P	K	Y	E	Q	G	G	N	I	V	D	I	D	F	D	S	V	P	Q	I	H	G	V31-37
D2	Q	N	K	G	N	Q	S	F	E	E	D	T	E	K	D	K	P	K	Y	E	H	G	G	N	I	I	D	I	D	F	D	S	V	P	H	I	H	G	
D3	F	N	K	H	T	E	I	I	E	E	D	T	N	K	D	K	P	S	Y	Q	F	G	G	H	N	S	V	-	D	F	E	E	D	T	L	P	K	V	
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FIG. 1

FIG. 2



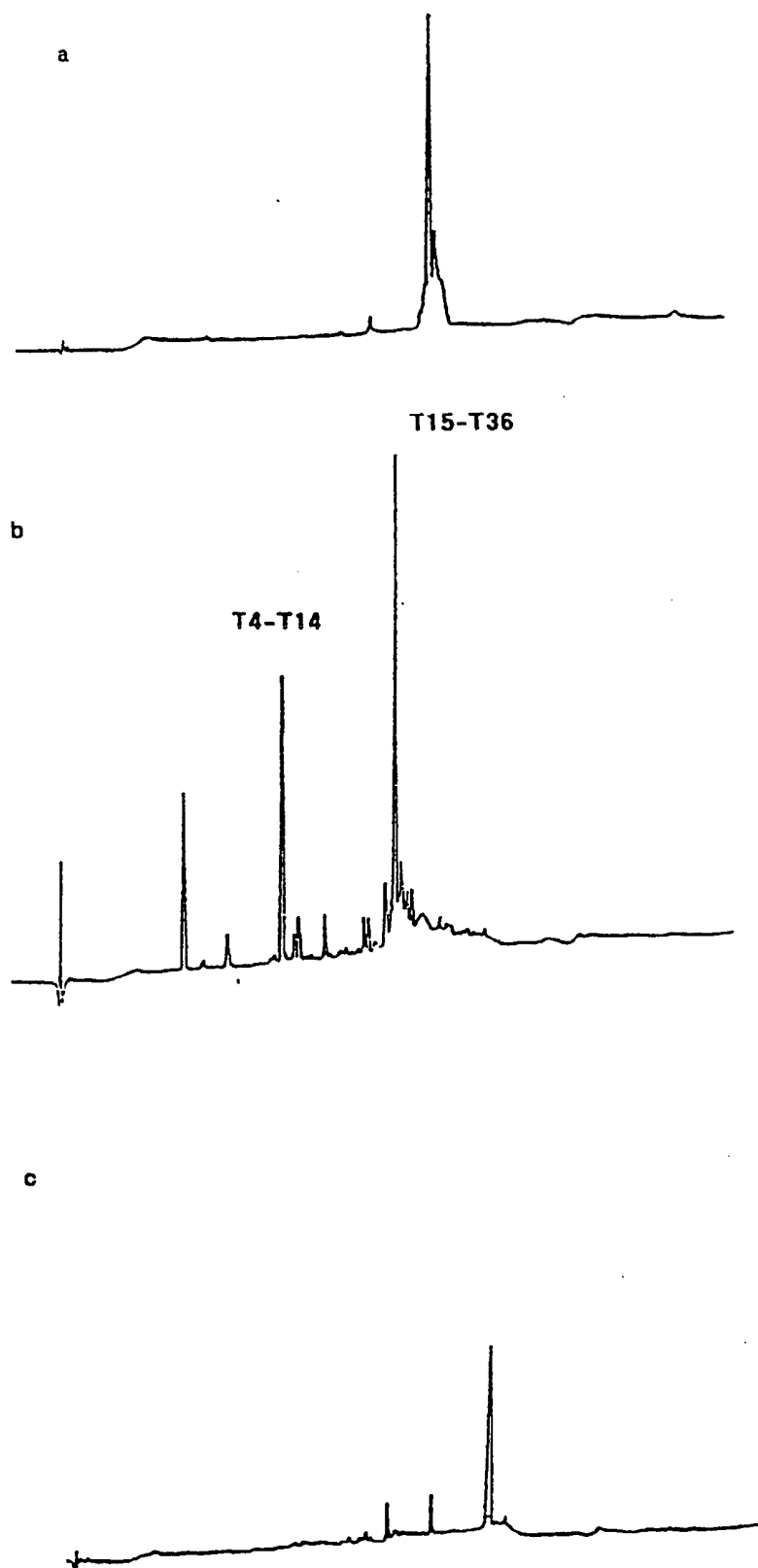


FIG. 3

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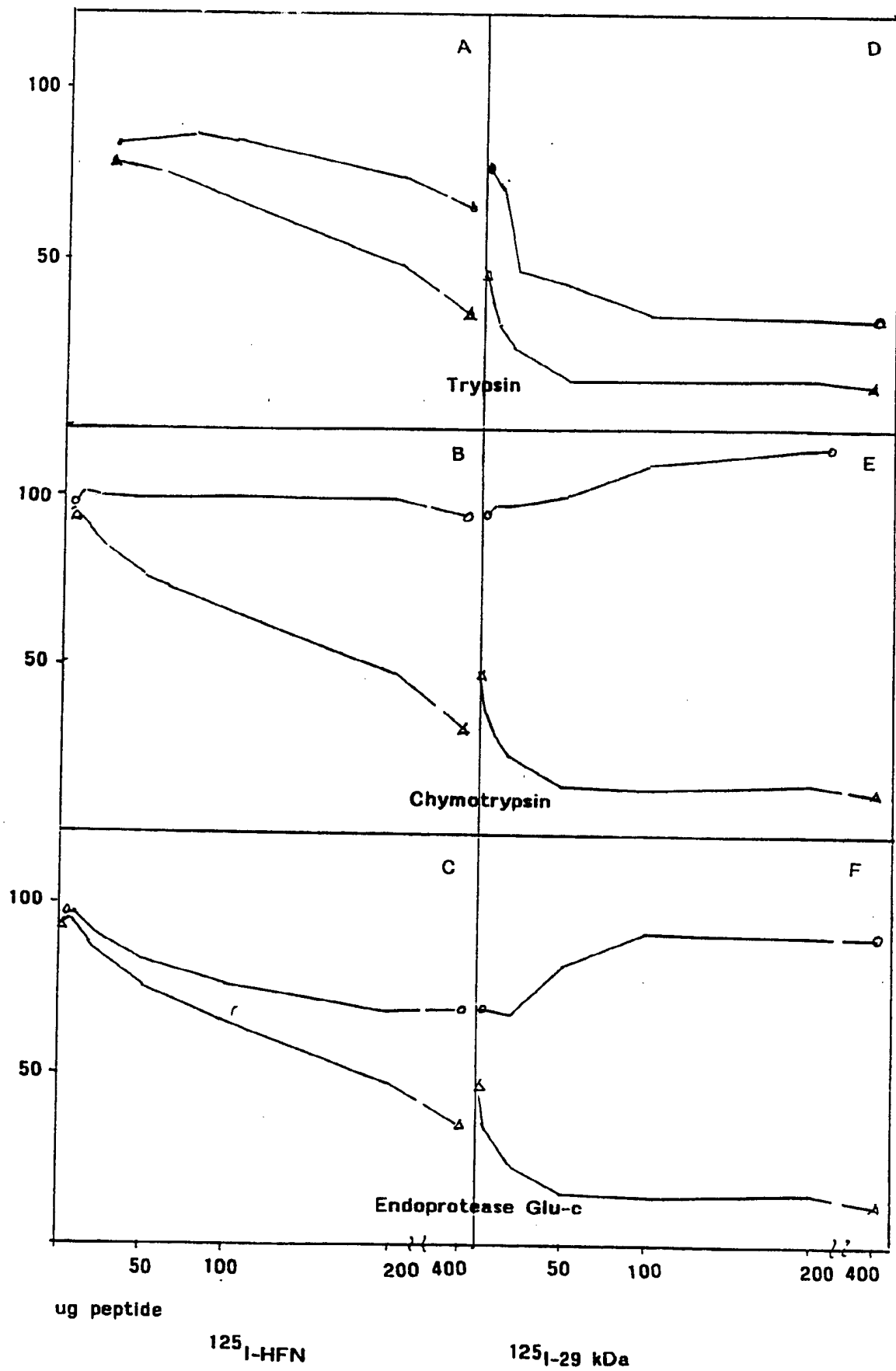


FIG. 4

SUBSTITUTE SHEET

## BINDING ACTIVITY (% of control)

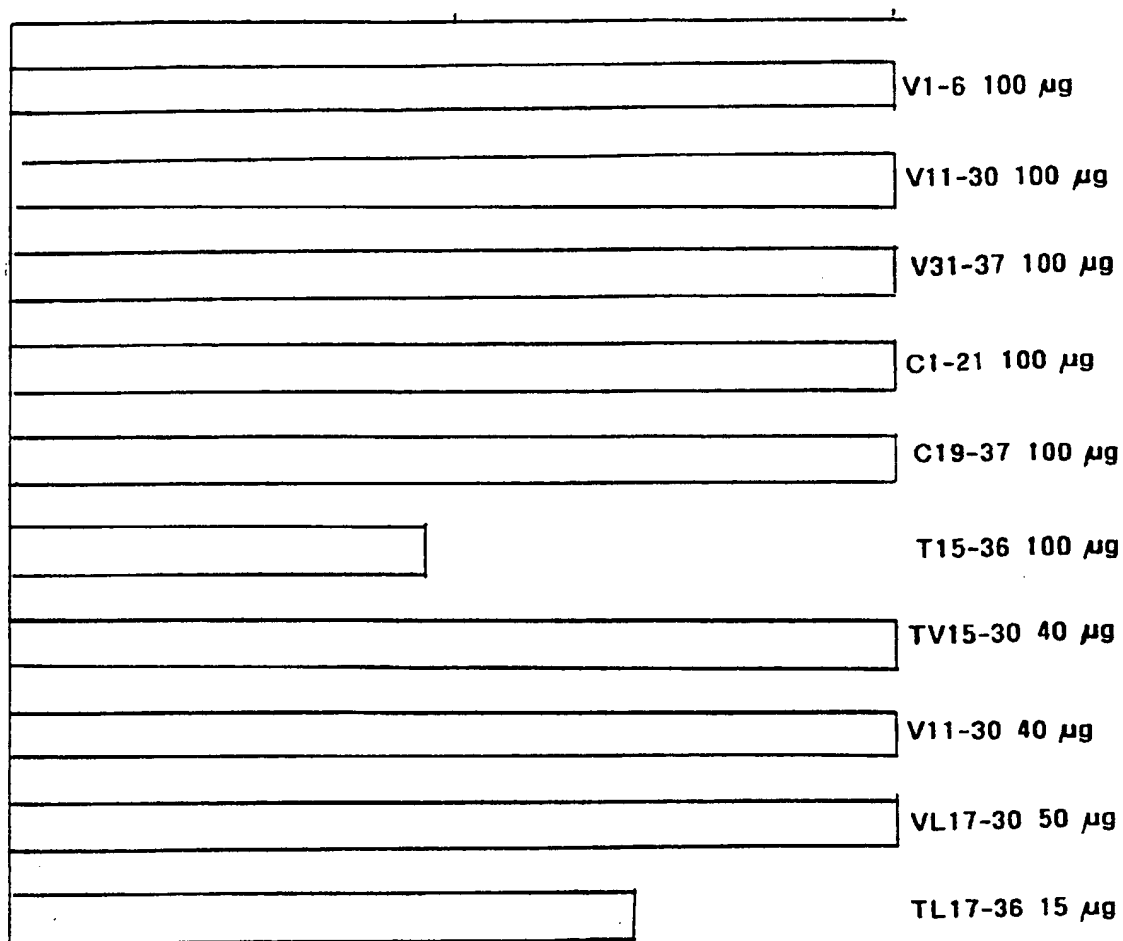


FIG. 5

Inhibition of  $^{125}\text{I}$ -HFN Binding to *S. aureus* 8235-4 by Synthetic Peptides

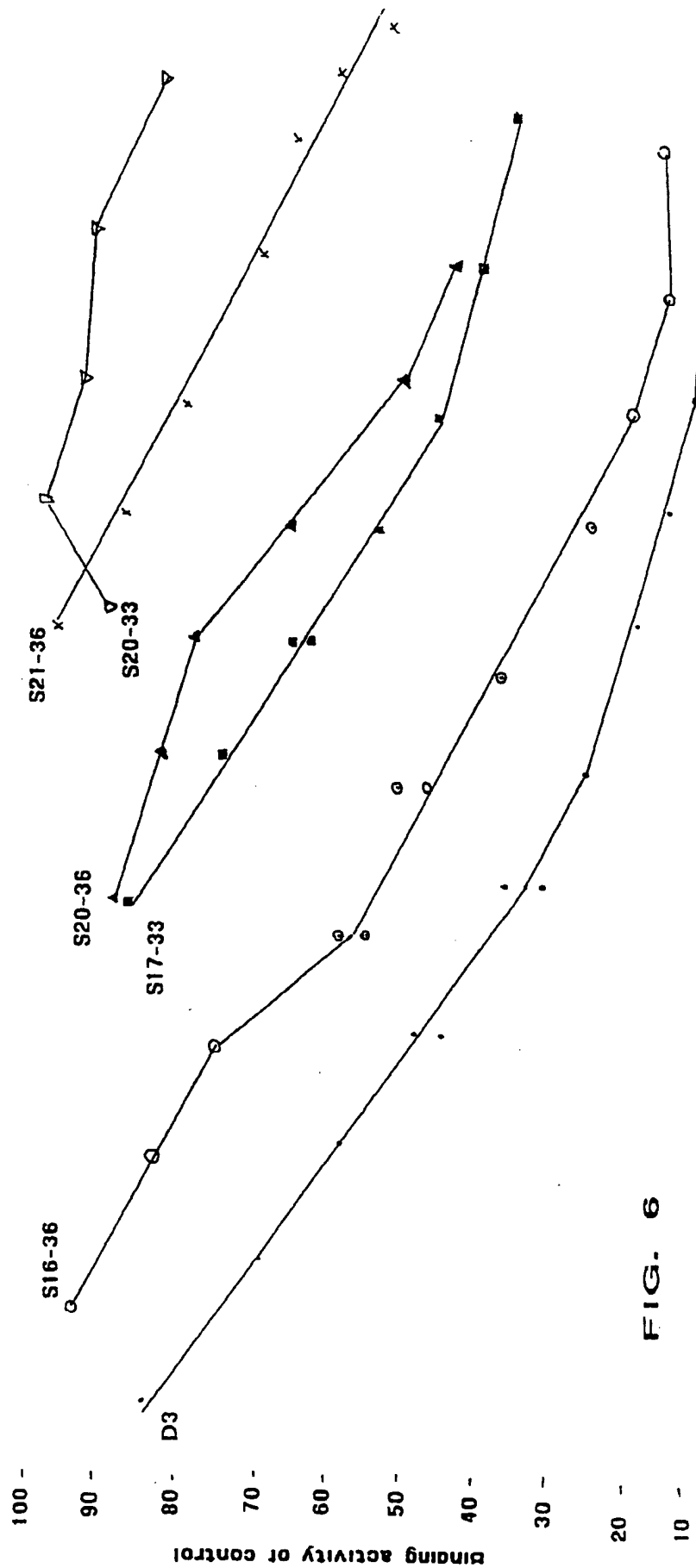


FIG. 6

# INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 91/00534

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 07 K 15/04, 7/08														
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border: 1px solid black; padding: 2px;">Minimum Documentation Searched<sup>7</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; border: 1px solid black;">Classification System</th> <th style="border: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; height: 40px; vertical-align: bottom;">IPC5</td> <td style="border: 1px solid black; vertical-align: bottom;">C 07 K</td> </tr> </table> <p style="font-size: small; text-align: center;">Documentation Searched other than Minimum Documentation to the extent that such Documents are included in Fields Searched<sup>8</sup></p> <p>SE,DK,FI,NO classes as above</p>			Classification System	Classification Symbols	IPC5	C 07 K								
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<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; font-size: small;">Category *</th> <th style="width: 60%; font-size: small;">Citation of Document,<sup>11</sup> with indication, where appropriate, of the relevant passages<sup>12</sup></th> <th style="width: 30%; font-size: small;">Relevant to Claim No.<sup>13</sup></th> </tr> </thead> <tbody> <tr> <td style="vertical-align: top;">P,X</td> <td>Dialog Information Services, File 154, Medline 85-91 Dialog accession no. 07698071, McGavin MJ et al: "Fibronectin binding determinants of the Staphylococcus aureus fibronectin receptor", &amp; J Biol Chem (UNITED STATES) May 5 1991, 266 (13) p8343-7 --</td> <td style="vertical-align: top; text-align: center;">1</td> </tr> <tr> <td style="vertical-align: top;">X</td> <td>EP, A2, 0294349 (ALFA-LAVAL AGRI INTERNATIONAL AB) 7 December 1988, see page 12, line 7 - line 8 --</td> <td style="vertical-align: top; text-align: center;">1</td> </tr> <tr> <td style="vertical-align: top;">X</td> <td>Dialog Information Services, File 154, Medline 85-91 Dialog accession no. 07409685, Raja RH et al: "Peptide analogs to a fibronectin receptor inhibit attachment of Staphylococcus aureus to fibronectin-containing substrates", &amp; Infect Immun Aug 1990, 58 (8) p2593-8 --</td> <td style="vertical-align: top; text-align: center;">1</td> </tr> </tbody> </table>			Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	P,X	Dialog Information Services, File 154, Medline 85-91 Dialog accession no. 07698071, McGavin MJ et al: "Fibronectin binding determinants of the Staphylococcus aureus fibronectin receptor", & J Biol Chem (UNITED STATES) May 5 1991, 266 (13) p8343-7 --	1	X	EP, A2, 0294349 (ALFA-LAVAL AGRI INTERNATIONAL AB) 7 December 1988, see page 12, line 7 - line 8 --	1	X	Dialog Information Services, File 154, Medline 85-91 Dialog accession no. 07409685, Raja RH et al: "Peptide analogs to a fibronectin receptor inhibit attachment of Staphylococcus aureus to fibronectin-containing substrates", & Infect Immun Aug 1990, 58 (8) p2593-8 --	1
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<div style="display: flex; justify-content: space-between; font-size: small;"> <div style="width: 45%;"> <p>* Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p> </div> </div>														
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border: 1px solid black; padding: 5px;">           Date of the Actual Completion of the International Search  <b>6th November 1991</b> </td> <td style="width: 50%; border: 1px solid black; padding: 5px;">           Date of Mailing of this International Search Report  <b>1991 -11- 11</b> </td> </tr> <tr> <td style="border: 1px solid black; padding: 5px;">           International Searching Authority  <div style="text-align: center; margin-top: 10px;"> <b>SWEDISH PATENT OFFICE</b> </div> </td> <td style="border: 1px solid black; padding: 5px;">           Signature of Authorized Officer  <div style="text-align: center; margin-top: 10px;">   <b>Mikael Bergstrand</b> </div> </td> </tr> </table>			Date of the Actual Completion of the International Search <b>6th November 1991</b>	Date of Mailing of this International Search Report <b>1991 -11- 11</b>	International Searching Authority <div style="text-align: center; margin-top: 10px;"> <b>SWEDISH PATENT OFFICE</b> </div>	Signature of Authorized Officer <div style="text-align: center; margin-top: 10px;">   <b>Mikael Bergstrand</b> </div>								
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	<p>Proc. Natl. Acad. Sci., Vol. 86, January 1989 C signäs et al.: "Nucleotide sequence of the gene for a fibronectin-binding protein from Staphylococcus aureus: Use of this peptide sequence in the synthesis of biologically active peptides", see page 699 - page 703 see fig. 3 p. 702</p> <p>-- -----</p>	1



ON INTERNATIONAL PATENT APPLICATION NO. PCT/SE 91/00534

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0294349	88-12-07	AU-D- JP-A- SE-A-	1691588 2154689 8702272
			88-12-01 90-06-14 88-12-02